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Robustness of larval development of intertidal sea urchin species to simulated ocean warming and acidification

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ABSTRACT

Ocean warming and acidification are the two most significant side effects of carbone dioxide emissions in the world's oceans. By changing water, temperature and pH are the main environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates. This study evaluated the combined effects of predicted high temperature levels, and predicted low pH values, on fertilization and early development stages of the sea urchins *Arbacia lixula, Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*. Twelve treatments, combining different temperatures (19, 21, 23 and 25 °C) and pH values (8.1, 7.7 and 7.4 units), were tested in laboratory experiments. All of the tested temperatures and pH values were within the open coast seawater range expected within the next century. We examined fertilization rate, cleavage rate, 3-day larvae survival, and development of the different sea urchin species at set time intervals after insemination. Our results highlight the susceptibility of subtidal species to environmental changes, and the robustness of intertidal species to ocean warming and acidification.

1. Introduction

Anthropogenic modification of the environment has triggered global climate change processes (IPCC, 2014). The partial pressure of CO_2 (pCO_2) has increased from 267 to 400 ppm, since the beginning of the Industrial Revolution (IPCC, 2014). Ocean warming is a direct effect of greenhouse gas emissions (Harley et al., 2006). As a result, it is thought that sea surface temperature (SST) will rise by 2–4.5 °C by the end of 21st century (IPCC, 2014). However, not all anthropogenic CO_2 accumulates in the atmosphere; the oceans are thought to have taken up approximately 25% of the carbon generated by human activities since 1800 (Sabine et al., 2004). In seawater, an increase in absorption of atmospheric CO_2 leads to modification of the carbonate system, causing a decrease in ocean pH, known as ocean acidification. Since anthropogenic activities have caused a massive flux of extra CO_2 into the atmosphere, more CO_2 has also dissolved into the oceans. As a result, mean surface ocean pH decreased by approximately 0.1 units between pre-industrial times and the end of the 20th century; a rate of acidification not otherwise seen within the last 60 million years (Caldeira and Wickett, 2005). A further decrease of approximately 0.4 units is expected by the end of the 21st century (Gattuso and Hansson, 2011; Turley et al., 2013). Ocean warming and acidification are causing substantial changes to marine physics, chemistry and biology, in ways that we are only beginning to understand (Turley et al., 2013).

Temperature and pH are two of the most important factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates (Doney et al., 2009). In echinoderms, temperature and pH have effects on several physiological responses, including respiration, feeding, fertilization and larval development (see reviews by Byrne et al., 2013a; Byrne and Przeslawski, 2013; Dupont and Thorndyke, 2013), as well as many genetic processes (Stumpp et al., 2011; Foo et al., 2012). Studies of natural gradients in pCO_2 show that some sea urchin species (e.g. *Arbacia lixula*) are more resilient to hypercapnia than others (e.g. *Paracentrotus lividus*) (Calosi et al., 2013; Small et al., 2015). However, few studies have tested the combined effects of temperature and pH, and those that have, produced contrasting findings (Byrne et al., 2009; Sheppard-Brennand et al., 2010; Byrne et al., 2010a; b; Ericson et al., 2012; Foo et al., 2012; Byrne et al., 2013b; Padilla-Garmiño et al., 2013; Gianguzza et al., 2014; Hardy and Byrne, 2014; Stavroff, 2014; García et al., 2015). More research is therefore needed, to determine the effects of these two stressors on different species.

Responses to climate change stressors seem to be highly species-specific, even in closely related taxa, and effects on fitness-related parameters can be either negative or positive (Wittmann and Pörtner, 2013). However, the habitat of the species seems to play an important

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role. It was recently hypothesized that scenarios, outside of present range natural environmental variability (Dorey et al., 2013), will induce more detrimental effects than conditions already experienced within the present natural range. Species that are regularly exposed to a high level of environmental variability, could therefore withstand future climate change scenarios better than those inhabiting more stable environments (Moulin et al., 2011; Foo et al., 2012; see Byrne et al., 2013a and Camp et al., 2018 for review). Those habituated to a wide range of temperature and pH scenarios already, are expected to possess greater resilience to seawater warming and/or acidification (Melzner et al., 2009; Talmage and Gobler, 2009, 2011; Matson et al., 2012; Wolfe et al., 2013). Prime examples are intertidal and invasive species (Hall-Spencer and Allen, 2015). Intertidal organisms show ecological strategies that enable them to survive in coastal areas, where environmental stress and disturbances are frequent. Their window of thermal tolerance is broad, which suggests significant phenotypic plasticity (Catarino et al., 2012; Calosi et al., 2013). Sea urchins living in extreme environmental conditions, have the ability to acclimatise to fluctuations in many seawater parameters, which means they are already partially equipped to cope with a range of environmental stresses (Hall-Spencer et al., 2008; Catarino et al., 2012). Hence, in our study, we have chosen to investigate the effects of temperature and pH on several different sea urchin species – two of which occupy habitats predominantly within the intertidal zone.

The Canary Islands are located within the subtropical region of the Eastern Atlantic Ocean. Tenerife is one of the central islands of the Archipelago, located at 28° N 16° W. In this area, there are four main species of sea urchins sharing the coastal ecosystems: *Arbacia lixula* (Linnaeus, 1758), *Paracentrotus lividus* (Lamarck, 1816), *Sphaerechinus granularis* (Lamarck, 1816) and *Diadema africanum* (Rodríguez et al., 2013). Each of these species has different environmental preferences (Fig. 1), and some of them, such as *P. lividus*, are at the geographical limits of their distribution. Studying organisms in an environment close to their geographic limit provides an opportunity to assess their potential adaptation and/or selection capabilities; because these species are already acclimatising to temperature and pH conditions at the limit of what they can physiologically withstand.

Our study assessed the combined effects of ocean warming and ocean acidification on: (1) fertilization and cleavage rate; and (2) early larval survival, growth and development, of the four main shallow-water species of sea urchins in the Canary Islands. We hypothesized that intertidal species (*A. lixula* and *P. lividus*), would be more resilient to climate change processes than subtidal species that live in a more stable environment (*S. granularis* and *D. africanum*).

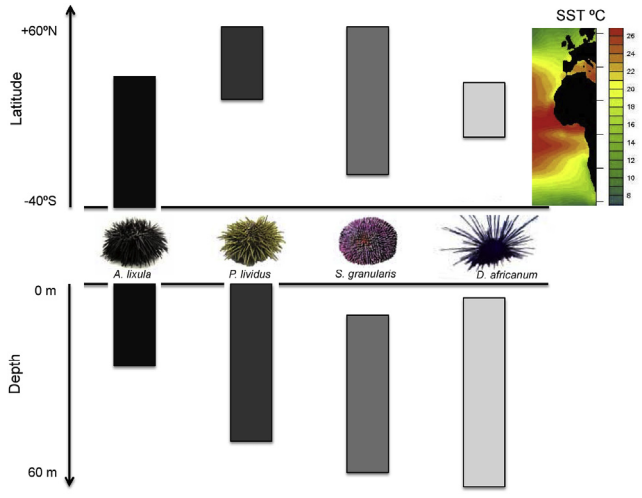

Fig. 1. Latitude and depth range of the studied sea urchin species (Source: http//www.marinespecies.org).

2. Materials and methods

2.1. Seawater parameters

Four different seawater temperature, and three different pH levels, were used in combination in this laboratory experiment. The temperature treatments were representative of the full range of water temperatures found in the Canary Islands within its current annual cycle. In addition, one higher temperature was incorporated, that has never been recorded around the Canary Islands to date, but which the IPCC (2014) predicts will be within the normal summer water temperature range by the year 2100. Overall the temperatures tested were: 19 °C (SST in spring in the Canary Islands); 21 °C (an exceptionally high value for March, but more typical SST in early summer); 23 °C (normal SST in summer, also predicted during spring by the year 2100, IPCC, 2014); and 25 °C (predicted temperature for the summer period by 2100, IPCC, 2014). Within each temperature treatment, three different treatments of pH were tested: pH 8.1 (control treatment; present average pH); pH 7.7 (present extreme of the natural variability, also average pH predicted for 2100 (IPCC, 2014)); and pH 7.4 (extreme of natural variability predicted by 2100 (Caldeira and Wickett, 2005)). In total, twelve different temperature-pH scenarios were tested.

To keep temperature conditions constant, thermostat coolers and heaters (EHEIM AQUATICS, 50 W) were used. In order to control pH, we used a computerised control system (AquaMedic) that regulated pH, by bubbling pure CO_2 directly into the water (resolution ± 0.01 pH units). In the three-day experiment, seawater was changed once on day 2. Temperature and pH were monitored using a Metrohm mobile meter, with a Primatrode NTC IP pH electrode, and temperature sensor. Salinity was measured using a handheld conductivity meter (COND 315i). Seawater total alkalinity (TA) was measured for each treatment, with a Titration system. PCO_2, calcite saturation state (Ωc), and aragonite saturation state (Ωa), were calculated from TA and pH using the software CO_2sys 2011 (Lewis and Wallace, 1998). Calculations were based on a set of constants, K1 and K2, following Mehrbach et al. (1973) (refit by Dickson and Millero, 1987).

Experiments were conducted with filtered seawater, which was purified within a recirculating system. The system used DRYDEN AQUA active filter media (AFM) bio-crystals; 50 µm, 10 µm and 1 µm UNICEL polyamide paper filters; and a UV-C AQUAEL 11W filter.

2.2. Animal collection and bioassays

Mature specimens of the sea urchin species *Arbacia lixula, Paracentrotus lividus, Sphaerechinus granularis and Diadema africanum* were collected by scuba divers, from subtidal rocky shores. Individuals were collected in 2013, during the spawning period for each species: summer for *A. lixula* (Fénaux, 1968; Wangensteen et al., 2013); late winter for *P. lividus* (Girard et al., 2008), spring for *S. granularis* (Guillou and Michel, 1993) and summer for *D. africanum* (Hernández et al., 2011). All collections were made from a site on the east coast of Tenerife (28°16′07″N, 16°36′20″W). Each species was tested in all twelve of the different temperature-pH scenarios.

Animals were induced to spawn by injection with 2 ml of KCl (0.5 M), through the peristomial membrane. Four males and five females of each species were randomly selected (Evans and Marshall, 2005). Gametes were mixed before being put in contact with gametes of the opposite sex. Sperm was collected dry and kept on ice until usage, and eggs were collected in filtered seawater. The same number of eggs was added to each of 144 separate experiment pots for each species. The appropriate quantity of sperm was then added to achieve the desired egg to sperm ratio of 1:1500. To carry out fertilization essays, eggs and sperm were added together in pots with 100 ml of filtered seawater. The temperature and pH of the filtered seawater was adjusted prior to its addition (twelve different combinations). To assess fertilization and early development, pots were incubated for different lengths of time

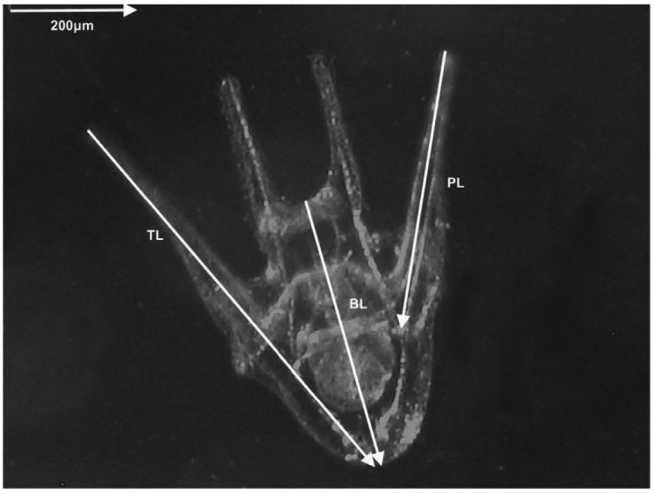

Fig. 2. Morphometric measurements taken for each three-day larva of the sea urchins *Arbacia lixula, Paracentrotus lividus*, *Sphaerechinus granularis and Diadema africanum* in laboratory experiments: Body length (BL), total length (TL) and post oral arm length (PL).

after insemination: 15, 105 and 210 min, and 3 days. For all combinations of temperature, pH and time after insemination, 3 replicates were performed. Development was halted by adding 1 ml of buffered formaldehyde (4%). Total fertilization rate was checked by counting the number of fertilized eggs in aliquots of 0.2 ml (three aliquots per replicate). To evaluate cleavage rate we used only the 15, 105 and 210 min after insemination samples. We estimated the number of eggs in different stages of cell division: non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells. To carry out this evaluation we observed three aliquots of 0.2 ml for each replicate, under a microscope.

Larval survival, growth and development were assessed in the three-day samples. Counting was done by taking 3 aliquots of 1 mL from each replicate pot. In total 10 larvae were photographed and measured from each replicate, using ImageJ software (Rasband, 1997–2012). Measurements of body length (BL), total length (TL) and post oral arm length (PL) were taken (Fig. 2). Overall, 30 larvae were measured and photographed for each combination of temperature and pH treatments.

2.3. Data analysis

In order to assess the combined effects of seawater temperature and pH on fertilization rate, and to compare the different species, data were analysed by means of a four-way permutational analysis of variance (PERANOVA) (Anderson, 2001). A four-way permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to analyze the combined effects of temperature and pH on cleavage rate, including the five stages of division (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells). In both cases, four-way designs were conducted with species (4 levels), temperature (4 levels), pH (3 levels) and time after insemination (3 levels) used as fixed factors.

To evaluate the combined effects of temperature and pH on larval survival in the studied species, data were analysed by means of a three-way permutational ANOVA. In order to analyze morphometric variables (BL, TL, PL) in three-day larvae development data, a three-way PERMANOVA was performed. In each case, three-way designs were carried out using species (4 levels), temperature (4 levels), and pH (3 levels), as fixed factors.

In all analyses of variance, significant terms were examined using *a posteriori* pairwise comparisons by permutations (Anderson, 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws, from the asymptotic permutation distribution. Principal Coordinates Analysis (PCO) ordinations of cleavage rate and three-day larvae development data were used to identify similarities between observations. All statistical analyses were carried out using PRIMER 6 & PERMANOVA + v. 1.0.1 software.

3. Results

The physico-chemical parameters of seawater, as measured during the experiments, are given in Table 1. The pCO_2 increased at low pH in all temperature treatments, while saturation levels of Ω_c and Ω_a decreased at low pH.

When analysing fertilization rate, a significant interaction of factors 'Species x Temperature x pH x Time' was found (Table 2A). In each combination of temperature and pH conditions, there were different responses for each of the studied species, at each of the different times after insemination. A *posteriori* pairwise test for *Arbacia lixula,* revealed a significant decrease in fertilization rate at low pH, across all temperatures, at 15 min after insemination. The same trend was significant at all time points at the lowest temperatures, but not at the highest temperatures (Fig. 3a). Non-significant differences were found in fertilization rate of *Paracentrotus lividus,* except in samples 15 min after insemination at 21 °C when fertilization rate was significantly higher at pH 7.7 compared to pH 8 (Fig. 3b; Supplementary material 1, 2). *Sphaerechinus granularis* showed a significant increase in fertilization rate with time, especially at the lowest temperatures (19 and 21 °C) (Fig. 3c; Supplementary material 1, 2). In *Diadema africanum* samples, fertilization rate significantly decreased with decreasing pH, and increased with increasing temperature, at 105 and 210 min after insemination (Fig. 3d; Supplementary material 1, 2). There were significant differences in fertilization rate between the species, particularly in the case of *D. africanum*, which exhibited the lowest fertilization percentages (Fig. 3; Supplementary material 3).

Results of the PERMANOVA analysing cleavage rate, showed a significant interaction of factors 'Species x Temperature x pH x Time' (Table 2B), indicating that the effects of temperature and pH, on the rate at which eggs reached different stages of cleavage, varied significantly with time after insemination. Pairwise comparisons in *A. lixula*, showed that the proportion of non-cleaved eggs, or eggs in early stages of development (with only the fertilization membrane, or divided into 2 cells) was greater at low pH. This pattern was significant at all temperature and times after insemination (Fig. 4a; Supplementary material 4). Also found with *A. lixula*, at higher seawater temperature, there was a greater proportion of eggs in the advanced stages of cleavage (eggs divided into > 4 cells), but only in treatments at control pH, and only at 210 min after insemination (Fig. 4a; Supplementary material 5). With *P. lividus*, the lower the seawater pH, the greater the proportion of non-cleaved eggs or eggs in early stages (with only the fertilization membrane or divided into 2 cells). This pattern was seen at all time points, but only at temperatures of 19 °C and 21 °C (Fig. 4b; Supplementary material 4). At 210 min after insemination, higher seawater temperatures (23 °C and 25 °C) corresponded with a greater proportion of eggs in the advanced stages of cleavage (eggs into > 4 cells), and pH had no influence on this result (Fig. 4b; Supplementary material 5). In the species *S. granularis*, the most advanced egg stages were found at 21 and 23 °C at pH 8.1. The greatest proportion of non-cleaved eggs or eggs in early stages of development (cells with fertilization membrane or divided into 2) coincided with the highest temperature (25 °C) and the lowest pH conditions (Fig. 4c; Supplementary material 4, 5). Amongst *D. africanum,* the proportion of eggs in advanced stages of development (> 4 cells) was significantly lower at low pH (7.7 and 7.4), at all temperatures. The most advanced stages (4 cells and > 4 cells) were found in samples kept at the highest temperature and pH (Fig. 4d; Supplementary material 4, 5).

Three-day larval survival was significantly affected by the

Table 1
Physico-chemical seawater parameters for each experimental treatment conducted with the different sea urchin species tested. T: seawater temperature (mean ± SD), pH: seawater pH (mean ± SD), S: salinity (mean ± SD), pCO_2: CO_2 partial pressure, TA: Total alkalinity, Ω_c: Saturation level of calcite, Ω_a: Saturation level of aragonite.

A. lixula experiment						
	19 °C			21 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	19.17 ± 0.15	19.27 ± 0.23	19.33 ± 0.06	21.20 ± 0.20	21.17 ± 0.23	21.23 ± 0.21
pH(n = 3)	8.18 ± 0.01	7.72 ± 0.01	7.40 ± 0.02	8.14 ± 0.01	7.67 ± 0.03	7.40 ± 0.01
S (n = 3)	36.70 ± 0.10	36.63 ± 0.06	36.63 ± 0.15	36.70 ± 0.10	36.63 ± 0.06	36.63 ± 0.15
pCO_2	394.40	1310.30	2858.40	448.20	1503.6	2903.30
TA (n = 1)	2375.52	2367.93	2365.54	2390.24	2358.43	2356.22
Ωc	4.64	1.85	0.93	4.58	1.76	0.98
Ωa	3.02	1.21	0.60	2.99	1.15	0.64
	23 °C			25 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	22.97 ± 0.06	22.93 ± 0.06	22,97 ± 0.06	25.13 ± 0.11	25.17 ± 0.06	25.13 ± 0.23
pH(n = 3)	8.08 ± 0.01	7.69 ± 0.02	7.41 ± 0.03	8.08 ± 0.01	7.67 ± 0.03	7.43 ± 0.00
S (n = 3)	36.77 ± 0.15	36.60 ± 0.26	36.70 ± 0.10	36.60 ± 0.46	36.73 ± 0.21	36.50 ± 0.40
pCO_2	540.60	1484.30	2918.60	560.60	1599.60	2865.40
TA (n = 1)	2421.03	2407.12	2384.65	2474.20	2421.31	2402.01
Ωc	4.35	1.97	1.07	4.70	2.04	1.20
Ωa	2.86	1.30	0.70	3.11	1.35	0.79
P. lividus experiment						
	19 °C			21 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	19.17 ± 0.15	19.27 ± 0.23	19.33 ± 0.06	21.23 ± 0.06	21.3 ± 0.00	21.23 ± 0.21
pH(n = 3)	8.09 ± 0.03	7.70 ± 0.05	7.40 ± 0.02	8.06 ± 0.01	7.67 ± 0.07	7.42 ± 0.02
S (n = 3)	36.63 ± 0.06	36.63 ± 0.11	36.63 ± 0.06	36.90 ± 0.00	36.80 ± 0.00	36.90 ± 0.00
pCO_2	899.10	1591.0	2582.6	863.10	1293.20	2800.80
TA (n = 1)	2106.40	2069.53	2069.94	2081.02	2048.21	2070.60
Ωc	2.03	1.21	0.80	2.21	1.54	0.79
Ωa	1.32	0.79	0.52	1.45	1.01	0.52
	23 °C			25 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	22.97 ± 0.06	22.92 ± 0.06	22,97 ± 0.06	25.20 ± 0.00	25.23 ± 0.11	25.07 ± 0.30
pH(n = 3)	8.05 ± 0.03	7.73 ± 0.02	7.43 ± 0.03	8.06 ± 0.05	7.66 ± 0.05	7.39 ± 0.03
S (n = 3)	36.77 ± 0.21	36.60 ± 0.26	36.70 ± 0.10	36.60 ± 0.53	36.73 ± 0.11	36.50 ± 0.50
pCO_2	1033.60	1265.70	2489.20	910.2	1367.1	2379.3
TA (n = 1)	2163.48	2121.63	2092.82	2220.17	2167.99	2148.82
Ωc	2.19	1.78	0.96	2.74	1.89	1.14
Ωa	1.44	1.17	0.63	1.81	1.25	0.76
S. granularis experiment						
	19 °C			21 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	19.27 ± 0.06	19.30 ± 0.10	19.33 ± 0.06	21.13 ± 0.23	21.23 ± 0.21	21.17 ± 0.15
pH(n = 3)	8.06 ± 0.04	7.66 ± 0.06	7.36 ± 0.05	8.09 ± 0.01	7.65 ± 0.02	7.37 ± 0.02
S (n = 3)	36.50 ± 0.10	36.77 ± 0.23	36.33 ± 0.15	36.83 ± 0.15	36.60 ± 0.10	36.60 ± 0.02
pCO_2	489.00	1342.70	2754.80	466.80	1432.90	2813.30
TA (n = 1)	2126.20	2096.35	2069.93	2181.52	2140.23	2127.23
Ωc	3.30	1.45	0.74	3.79	1.53	0.83
Ωa	2.15	0.94	0.48	2.48	1.00	0.54
	23 °C			25 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	22.93 ± 0.06	23.00 ± 0.00	22,97 ± 0.06	25.20 ± 0.00	25.23 ± 0.11	25.06 ± 0.30
pH(n = 3)	8.06 ± 0.05	7.70 ± 0.02	7.43 ± 0.04	8.02 ± 0.03	7.69 ± 0.04	7.40 ± 0.03
S (n = 3)	36.70 ± 0.10	36.70 ± 0.10	36.70 ± 0.10	36.83 ± 0.15	36.73 ± 0.15	36.77 ± 0.15
pCO_2	518.10	1316.30	2539.30	592.80	1399.50	2829.1

(*continued on next page*)

Table 1 (*continued*)

A. lixula experiment						
	19 °C			21 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
TA (n = 1)	2203.48	2192.36	2179.13	2232.91	2229.36	2212.36
Ωc	3.79	1.84	1.02	3.79	1.96	1.04
Ωa	2.49	1.21	0.67	2.51	1.29	0.69
D. africanum experiment						
	19 °C			21 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	19.03 ± 0.25	19.17 ± 0.40	19.13 ± 0.38	21.20 ± 0.20	21.17 ± 0.23	21.23 ± 0.21
pH(n = 3)	8.10 ± 0.03	7.71 ± 0.05	7.41 ± 0.02	8.08 ± 0.01	7.68 ± 0.07	7.43 ± 0.02
S (n = 3)	36.70 ± 0.10	36.63 ± 0.06	36.63 ± 0.15	36.90 ± 0.10	36.80 ± 0.10	36.90 ± 0.10
pCO_2	456.40	1210.60	2465.60	511.80	1322.50	2311.60
TA (n = 1)	2215.50	2139.42	2096.50	2322.91	2132.44	2022.64
Ωc	3.70	1.63	0.83	3.98	1.63	0.90
Ωa	2.41	1.06	0.54	2.60	1.06	0.59
	23 °C			25 °C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n = 3)	23.03 ± 0.15	23.07 ± 0.21	23.00 ± 0.10	25.13 ± 0.11	25.17 ± 0.06	25.13 ± 0.23
pH(n = 3)	8.06 ± 0.03	7.74 ± 0.02	7.44 ± 0.03	8.07 ± 0.05	7.67 ± 0.05	7.40 ± 0.03
S (n = 3)	36.77 ± 0.21	36.60 ± 0.26	36.70 ± 0.10	36.60 ± 0.46	36.73 ± 0.21	36.50 ± 0.40
pCO_2	574.00	1203.6	2416.0	580.60	1486.90	2722.70
TA (n = 1)	2432.84	2213.46	2124.01	2492.34	2253.50	2125.14
Ωc	4.22	2.02	1.02	4.65	1.89	0.99
Ωa	2.77	1.33	0.67	3.07	1.25	0.66

Table 2
Results of (A) the four-way ANOVA analyzing fertilization rates; (B) the four-way PERMANOVA testing cleavage rate, including five studied variables (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells); (C) the three-way ANOVA analyzing larval survival; and (D) the three-way PERMANOVA testing 3-day larval development analysing morphometric variables (body length, total length and post oral arm length) of early stages of the studied sea urchin species. In the respective models the factors included are: Sp: species, T: temperature, pH, Ti: Time after insemination.

Source of variation	A. Fertilization Rate					B. Cleavage Rate				
	df	SS	MS	Pseudo-F	P (perm)	df	SS	MS	Pseudo-F	P (perm)
Sp	3	3.64E5	1.21E5	4488.90	0.001	3	8,28E5	2,76E5	1580,40	0.001
T	3	1136	378.68	13.99	0.001	3	30164	10055	57,58	0.001
pH	2	6570.10	3285.10	121.36	0.001	2	10358	5178,80	29,66	0.001
Ti	2	1.31E5	65656	2425.40	0.001	2	3,31E5	1,66E5	948,90	0.001
Sp*T	9	17014	1890.50	69.84	0.001	9	1,06E5	11824	67,71	0.001
Sp*pH	6	2045.70	340.96	12.59	0.001	6	9582	1597	9,14	0.001
Sp*Ti	6	73517	12253	452.64	0.001	6	3,91E5	65237	373,59	0.001
T*pH	6	458.81	76.469	2.82	0.014	6	8156,70	1359,40	7,78	0.001
T*Ti	6	9750.70	1625.10	60.03	0.001	6	93193	15532	88,95	0.001
pH*Ti	4	807.44	201.86	7.46	0.001	4	8241,9 0	2060,50	11,80	0.001
Sp*T*pH	18	1739.60	96.64	3.57	0.001	18	25580	1421,10	8,14	0.001
Sp*T*Ti	18	20390	1132.80	41.85	0.001	18	1,84E5	10250	58,70	0.001
Sp*pH*Ti	12	5060.40	421.70	15.58	0.001	12	20115	1676,20	9,60	0.001
T*pH*Ti	12	1830	152.50	5.63	0.001	12	17159	1430	8,19	0.001
Sp*T*pH*Ti	36	5760	160	5.91	0.001	36	48070	1335,30	7,65	0.001
Source of variation	C. Larval survival					D. 3 day Larval Development				
	df	SS	MS	Pseudo-F	P (perm)	df	SS	MS	Pseudo-F	P (perm)
Sp	3	5.51E6	1.84E6	2071.70	0.001	3	15,75	5,25	1141	0.001
T	3	50832	16944	19.10	0.001	3	1,84	0,61	133,57	0.001
pH	2	94204	47102	53.09	0.001	2	0,79	0,40	86,40	0.001
Sp*T	9	6.25E5	69447	78.27	0.001	9	6,21	0,69	150	0.001
Sp*pH	6	1.12E5	18667	21.04	0.001	6	0,49	8,26E-2	17,95	0.001
T*pH	6	10178	1696.30	1.91	0.065	6	0,49	8,17E-2	17,76	0.065
Sp*T*pH	18	72575	4032	4.54	0.001	18	0,58	3,21E-2	6,98	0.001

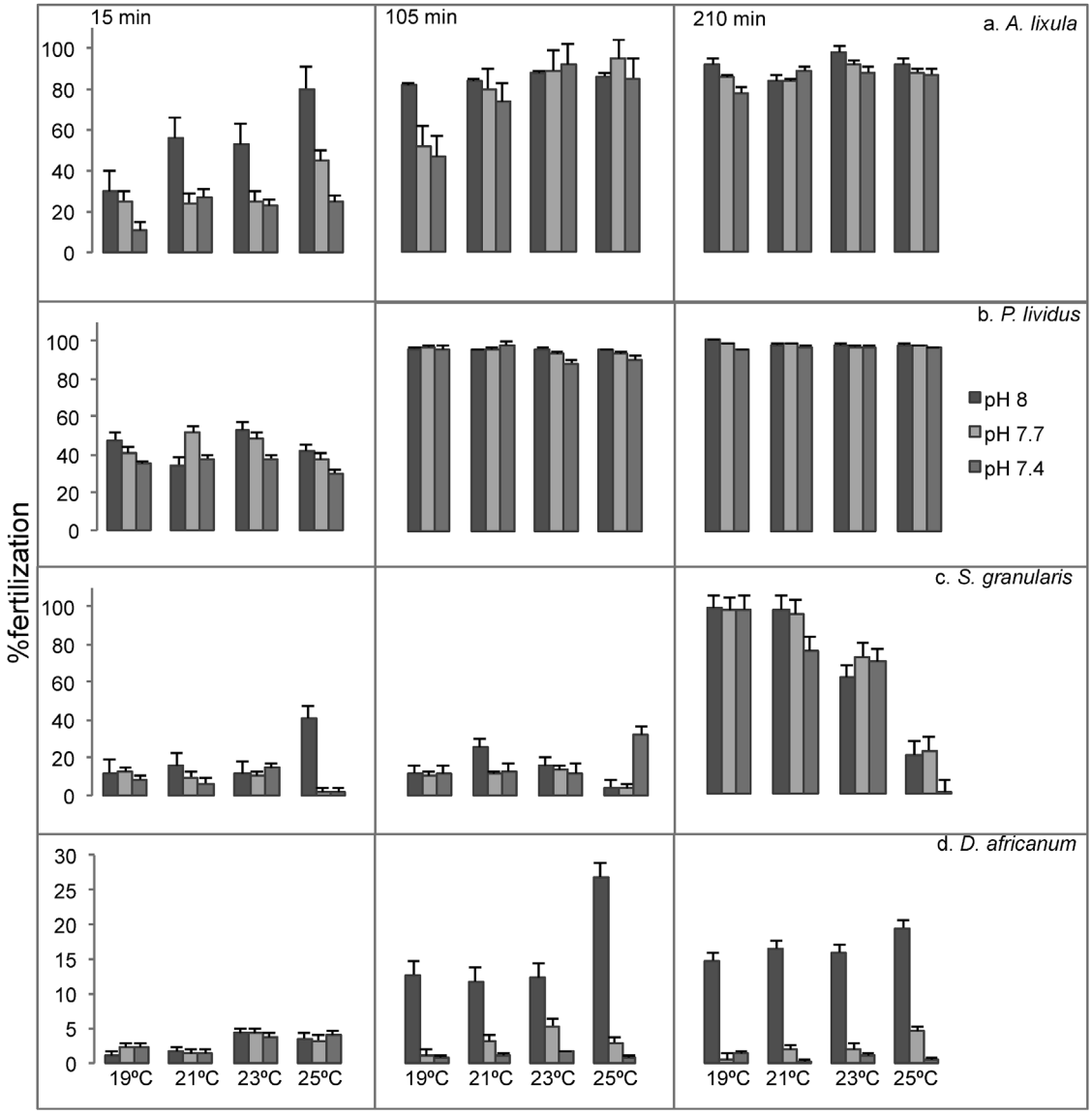

Fig. 3. Percentage of fertilized eggs (mean ± 27.50, 27.28, 33.47, 6.63) of the sea urchin species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis,* (d) *Diadema africanum* in laboratory experiments testing the combined effects of seawater temperature and pH in different times after insemination (15, 105 and 210 min).

interaction 'Species x Temperature x pH '(Table 2C). The density of *A. lixula* larvae was lower at low pH, at temperatures of 19, 21 and 23 °C. However, at 25 °C larval density was significantly higher in samples kept at pH 7.7. At all levels of pH, the density of larvae was significantly higher in the highest temperature conditions (21, 23 and 25 °C) (Fig. 5a; Supplementary material 6). Amongst *P. lividus,* larval survival decreased at low pH (7.7, 7.4) in all temperature treatments, except at 19 °C where the difference between pH 8.1 and 7.7 was not significant. At all levels of pH, larval density was lower at temperatures above 21 °C (Fig. 5b; Supplementary material 6). *S. granularis* larvae were highest in density at pH 7.7, at temperatures of 19 and 21 °C. This pattern was not the same at higher temperature, where the number of larvae per ml was negligible at all pH levels (Fig. 5c; Supplementary material 6). The density of *D. africanum* larvae was lower at low pH (7.7, 7.4) in samples kept at all temperatures. However, the number of larvae per ml did increase with increasing temperature, across all levels of pH (Fig. 5d; Supplementary material 6).

A significant interaction of 'Species x Temperature x pH ' (Table 2D) was found when assessing three-day larval morphology. This indicates that morphology is affected by both temperature and pH, but the effects are different for each of the four species. Pairwise comparisons in *A. lixula* showed that development of larvae was significantly higher at the highest temperatures (23, 25 °C), across all levels of pH (Fig. 6a; Supplementary material 7). In *P. lividus* samples, the lower the seawater pH, the less developed the larvae (at 19 and 21 °C). The most developed larvae were found at 21 °C in the control pH (Fig. 6b; Supplementary material 7). In the species *S. granularis*, larvae were significantly more developed at high pH (pH 8.1 and 7.7), but only at temperatures of 21 and 23 °C. The most developed larvae were found at 21 and 23 °C across all pH levels (Fig. 6c; Supplementary material 7). No measurements of *S. granularis* larvae could be made at 25 °C, because the number of larvae in the sample was too low. Amongst *D. africanum,* the most developed larvae were found at 25 °C and pH 8.1. Larval development decreased at lower temperature and lower pH (Fig. 6d; Supplementary material 7).

4. Discussion

The present study confirmed that responses to climate change stressors in the marine environment are highly species-specific. The

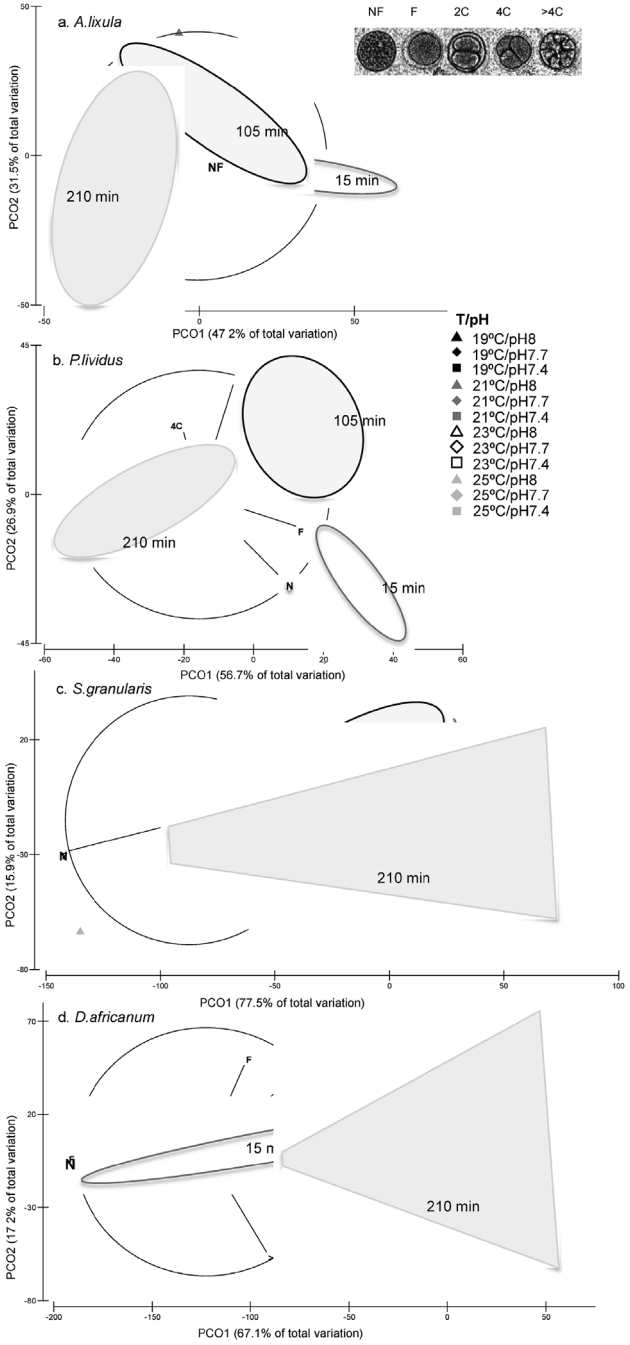

Fig. 4. PCO ordinations showing the combined effect of seawater temperature and pH on cleavage rate of the sea urchins species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis* and (d) *Diadema africanum*, at 15,105 and 210 min after insemination in laboratory experiments studying early development of the sea urchin. Vectors represent studied variables. NF: non-fertilized eggs, F: eggs only with the fertilization membrane, 2 C: eggs divided in 2 cells, 4 C: eggs divided in 4 cells, > 4C: eggs divided in more than 4 cells. Percentages of variation explained by each of the axes are given in brackets.

species tested seemed to be robust covering the present natural variability. However, subtidal species are more vulnerable to the environmental changes predicted to occur by the end of the century.

Fertilization rate in *Arbacia lixula* was lower at low pH, but the trend was only seen at low temperatures with time after insemination. Higher fertilization at higher temperatures (> 21 °C) has also been noted before in this species (Privitera et al., 2011). Though another study found no significant effects of temperature or pH (Gianguzza et al., 2014). *A. lixula* is a thermophilic species (Kempf, 1962; Guidetti and Dulcić 2007), which may explain why its fertilization rate performance is better under warmer conditions (with no effect of pH). The species may be robust enough to withstand the future-warming trend, as previously highlighted by (Wangensteen et al., 2013). Low pH caused a moderate delay on cleavage rate, at all temperatures. Larval survival was also generally lower at low pH, and higher at higher temperature. Wangensteen et al. (2013) also reported an increase in larval survival rates with rising temperature. In addition, larval development was greater at higher temperatures, under all pH conditions, which is consistent with previous studies (Privitera et al., 2011; Wangensteen et al., 2013). Therefore, *A. lixula* performed best overall at higher temperatures, and in some cases at lower pH. This suggests that *A. lixula* performance could be improved by near future climate change conditions in specific areas, as suggested by Gianguzza et al. (2014).

Paracentrotus lividus also showed great tolerance for warm and acidified waters. Previous studies have also found high fertilization success in this species (Moulin et al., 2011; Privitera et al., 2011; Martin et al., 2011; Cohen-Rengifo et al., 2013). However, cleavage rate was more sensitive than fertilization. Previous studies on this species have also noted a delay in cleavage rate at low pH and optimal temperatures (Moulin et al., 2011; Cohen-Rengifo et al., 2013). Though others found cleavage rate was only affected negatively under very low pH conditions (pH 7.0) (Martin et al., 2011). In our study, the negative effect of pH was neutralised by increasing the water temperature. However, this thermal tolerance was not apparent in the three-day larval survival where the negative effect of low pH increased at warmer temperatures. The density of larvae dropped at temperatures above 21 °C. Larval development was also stunted by low pH, being slightly ameliorated at higher temperatures. Similar delays in development of larvae have been reported for *P. lividus* previously (Moulin et al., 2011; Martin et al., 2011; Cohen-Rengifo et al., 2013). Other studies have also reported a strong influence of high temperature on the species after blastulation (Ericson et al., 2012; Foo et al., 2012; García et al., 2015). Martin et al. (2011) suggested that the species has high molecular plasticity, as a result of gene up-regulation, which allows growth and skeletal calcification to be maintained at low pH. Our results showed that exposing *P. lividus* to a scenario of low pH alongside high temperature, could cancel out some of the negative effects of ocean acidification on fertilization rate, cleavage rate and larval development. Similar trade-off between high temperature and low pH, has been found in recent studies (Sheppard-Brennand et al., 2010; Byrne et al., 2013b; García et al., 2015). A genetic adaptability has also been reported, showing that certain species have genotypes tolerant of increased temperature that are also tolerant of decreased pH (Foo et al., 2012). However, when considering larval survival, the combined effects of low pH and high temperature had deleterious impacts. This suggests the species has a narrower thermal tolerance after blastulation.

For *Sphaerechinus granularis,* fertilization rate was extremely impaired at higher temperatures, especially at 25 °C. Cleavage rate was effectively impaired by the most extreme combination of stressors (25 °C and pH 7.4). *S. granularis* is a temperate species, with a single spawning period in spring (Guillou and Michel, 1993; Guillou and Lumingas, 1998). In the Canaries, the population occupies mainly subtidal habitats, and densities are rarely high. (pers. obs.). At higher temperatures the density of larvae dropped and at 25 °C the number of larvae was too low to count accurately. These findings show the bad performance of early stages at high temperatures, suggesting a narrow threshold of the species to face global warming.

Diadema africanum was also significantly affected by low pH and high temperature. *D. africanum* is a thermophilic species that displays an annual reproductive cycle which peaks during summer in the Canary Islands, to coincide with the warmer SSTs (Hernández et al., 2011). This explains why its performance was best under warm conditions when at its optimal pH. However, at lower pH, its performance was unsuccessful

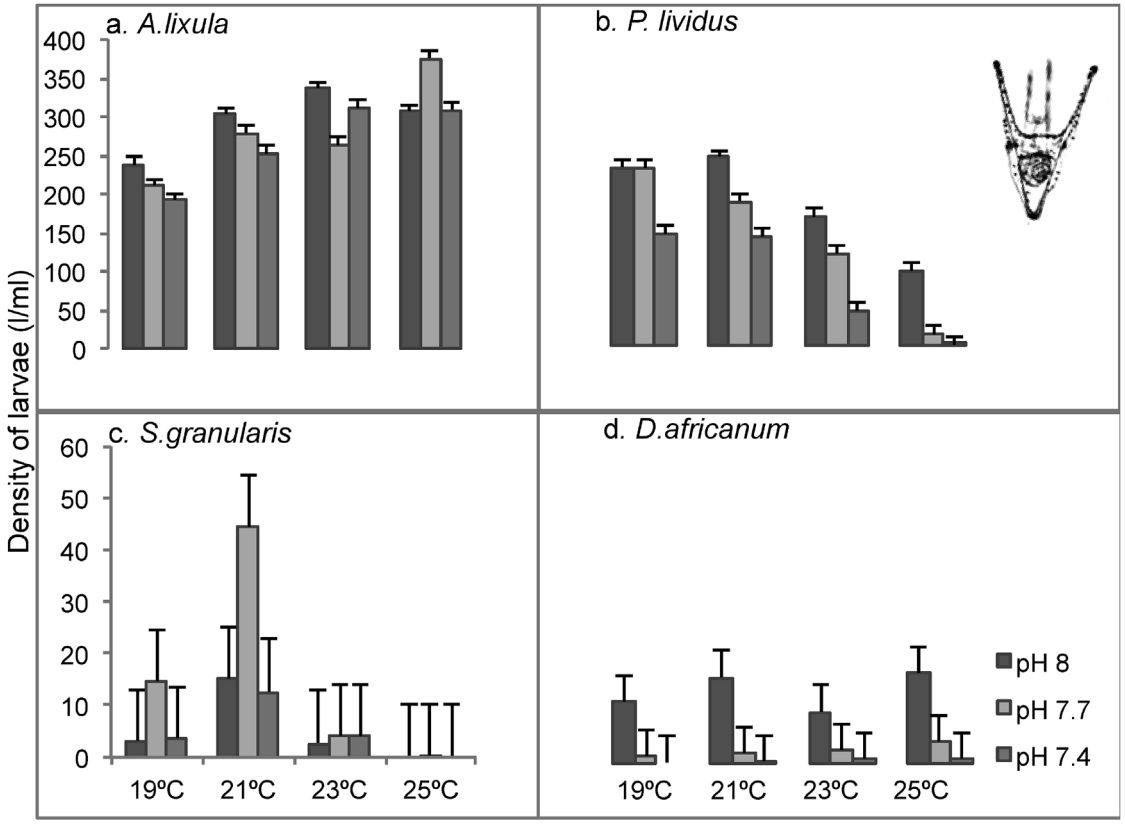

Fig. 5. Density of larvae (number of larvae per ml (mean ± SD)) of the sea urchin species (a) *Arbacia lixula* (mean ± 53.01), (b) *Paracentrotus lividus* (mean ± 82.08), (c) *Sphaerechinus granularis* (mean ± 12.52), (d) *Diadema africanum* (mean ± 6.39) at laboratory experiments testing the combined effects of seawater temperature and pH.

demonstrating that this is a likely vulnerable species under expected ocean acidification conditions. *D. africanum* is mainly a subtidal species (Hernández et al., 2006), used to a stable environment, which may explain its lack of pH tolerance (Byrne et al., 2010b; Moulin et al., 2011).

Exposing sea urchins to two environmental stressors simultaneously resulted in highly species-specific patterns. A broad thermo-tolerance of early stages of sea urchins has been shown by Byrne (2010), and it is well known that a moderate increase in temperature can improve fertilization rates (Hagström and Hagström, 1959; Mita et al., 1984). Some tolerate of acidification (~0,6 units) has also been shown previously (Kurihara and Shirayama, 2004; Byrne et al. 2009, 2010a), perhaps a result of the natural acidified environment to which gametes are exposed during reproduction (Holland et al., 1984). Both temperature and pH are harmless, until a sublethal threshold is reached. Their combined effect, in extreme conditions, may also have deleterious impacts. However, our study demonstrates that the effects of the interaction of climate change stressors differ among species identity and their life-history stages (see also reviews by Byrne, 2011; Byrne et al., 2013a; Dupont and Thorndyke, 2013).

Generally, temperature and pH had a greater impact on cleavage rate than fertilization rate. This can be explained by tolerance in fertilization, conveyed by maternal factors imprinted by ovary temperature (Fujisawa, 1995; Yamada and Mihashi, 1998), protective heat shock proteins (Hamdoun and Epel, 2007), and the low natural pH associated with echinoderm reproduction (Holland et al., 1984). There is strong evidence that adult thermal history influences thermal tolerance in fertilization due to adaptive phenotypic plasticity with respect to prevailing temperatures (O'Conner and Mulley, 1977; Fujisawa, 1995; Johnson and Babcock, 1994; Bingham et al., 1997). Also, the internal pH of activated sperm is ~7.6, and eggs also release acid at fertilization (Peaucellier and Doree, 1981; Holland and Gould-Somero, 1982); gametes are therefore made to withstand a certain level of pH variation. We observed a general trend towards delayed cleavage at low pH, as has been reported in the literature (Kurihara and Shirayama, 2004; Moulin et al., 2011; Martin et al., 2011; Foo et al., 2012; Ericson et al., 2012; Cohen-Rengifo et al., 2013). A postponement of cleavage could compromise subsequent developmental stages (O'Connor et al., 2007).

The occurrence of abnormal larvae has previously been found to increase at low pH (Moulin et al., 2011; Sheppard-Brennand et al., 2010; Martin et al., 2011; Cohen-Rengifo et al., 2013; Byrne et al., 2013a; Padilla-Garmiño et al., 2013). We also observed reduced size at low pH, among the four species we studied. Reduced size, or arm length, can have lethal consequences, since post-oral arms enable food uptake and swimming (Strathmann, 1971; Chan et al., 2011). Mortality can therefore be increased by starvation and susceptibility to predators. The planktonic stages of many echinoids are also synchronized with the availability of food-blooms, so a delay in development can cause a mismatch and increase susceptibility to starvation even further (Reitzel et al., 2004). Such decreases in larval survival, among the key sea urchin species, can have cascading effects (Clark et al., 2009) that change the structure and functioning of entire ecosystems. The effects of climate change on early larval development can also be explained at transcriptional responses level. It is thought that low pH can reduce body lengths as a result of impaired skeletogenesis (Padilla-Garmiño et al., 2013). Increases in temperature can increase expression of basic components of the cellular cytoskeleton, but low pH can decrease this expression (Padilla-Garmiño et al., 2013).

Our results suggest that intertidal species (*A. lixula* and *P. lividus*) may be less vulnerable to the effects of predicted climate change than the subtidal species (*S. granularis* and *D. africanum*), as hypothesized. Intertidal species have a greater capacity to cope with environmental change due to the variability in temperature and pH that they are

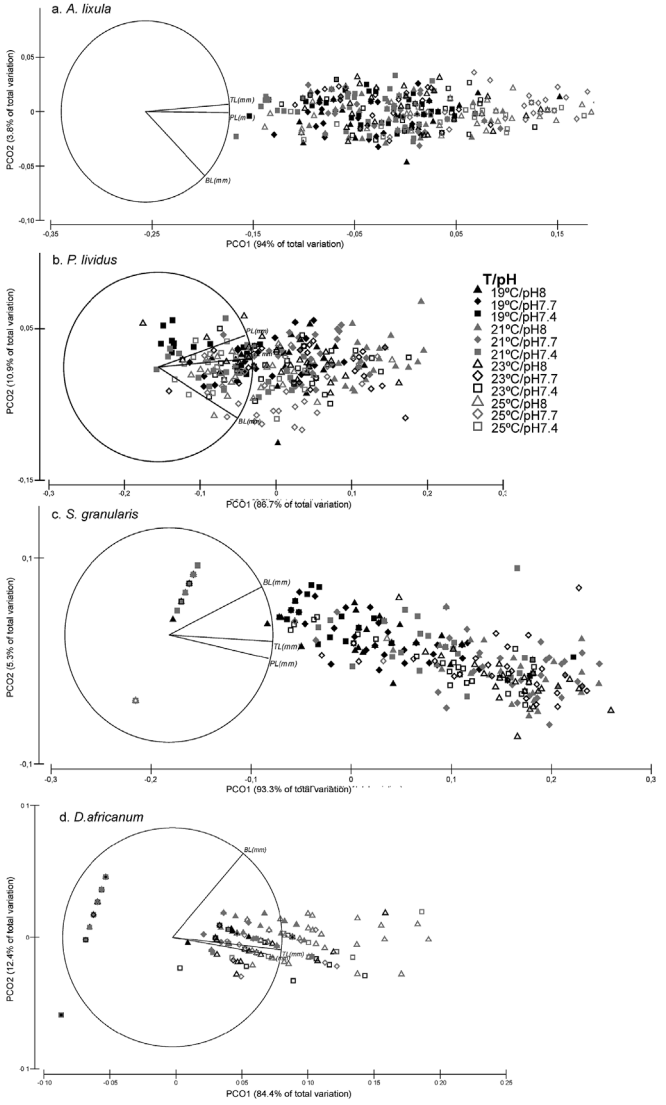

Fig. 6. PCO ordinations showing the combined effect of sweater temperature and pH levels on larval development at 3 days after insemination at laboratory experiments of the sea urchin species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis* and (d) *Diadema africanum*. Vectors represent studied morphological variables: TL: total length, BL: body length, PL: post-oral arm length. Percentages of variation explained by each of the axes are given in brackets.

already exposed to. Based on a recent study, one of these intertidal species may have an advantage over the other. Calosi et al. (2013) found that *A. lixula* was more abundant than *P. lividus* at low pH, noting that the former has a greater ability to regulate its extracellular fluid under elevated $pCO_{2\ conditions}$. Increases in densities of *A. lixula* populations could have serious consequences for the structure and functioning of the ecosystem (Wangensteen, 2013; Gianguzza et al., 2014). Subtidal species are thought to be less resilient to changes in temperature and/or acidification (Melzner et al. 2009, 2013; McElroy et al., 2012; Nguyen et al., 2012; Kelly et al., 2013). Of the four species investigated, *D. africanum* exhibited the lowest fertilization rates, and the percentage of fertilized eggs was dramatically impaired at low pH. Due to its highly specialized reproductive strategy, which is also greatly affected by environmental factors (Hernández et al., 2011), *D. africanum* is particularly vulnerable to climate change processes. *S. granularis* was also significantly impacted by environmental stress It is likely that survival of its populations will be constrained by the water temperature extremes predicted for the end of the century. We hypothesize that synergistic stressors like ocean acidification have the potential to narrow the thermal windows in the species, being highly dependent upon thermal conditions. Depending on the optimal temperatures of the species, warming may lead to increased resilience to ocean acidification at the lower end of the optimal temperature range, but it can enhance sensitivity when temperature is closer to the upper limit of tolerance.

This study highlights the importance of assessing the combined effects of stressors, and looking at their impacts across different life-history stages to understand the potential impacts of climate change processes on marine organisms. Intertidal species seem to be more resilient to near-future climate change scenarios than subtidal species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2018.04.011.

References

Anderson, M.J., 2001. Permutation tests for univariate or multivariate analysis of variance and regression. Can. J. Fish. Aquat. Sci. 58, 626–639.
Bingham, B.L., Bacigalupi, M., Johnson, L.G., 1997. Temperature adaptations of embryos from intertidal and subtidal sand dollars (*Dendraster excentricus*, Wschscholtz). Northwest Sci. 71, 108–114.
Byrne, M., Ho, M., Selvakumaraswamy, P., Nguyen, H., Dworjanyn, S., Davis, A., 2009. Temperature, but not pH, compromises sea urchin fertilization and early development under near-future climate change scenarios. Proc. Soc. Lond B Biol. Sci. 279, 1883–1888.
Byrne, M., 2010. Impact of climate change stressors on marine invertebrate life histories with a focus on the Mollusca and Echinodermata. In: Yu, Y., Henderson-Sellers, A. (Eds.), Climate Alert: Climate Change Monitoring and Strategy. University of Sydney Press, Sydney, pp. 142–185.
Byrne, M., Soars, N., Selvakumaraswamy, P., Dworjanyn, S., Davis, A., 2010a. Sea urchin fertilization in a warm, acidified and high pCO_2 ocean across a range of sperm densities. Mar. Environ. Res. 69, 234–239.
Byrne, M., Soars, N., Ho, M., Wong, E., McElroy, D., Selvakumaraswamy, P., Dworjanyn, S., Davis, A., 2010b. Fertilization in a suite of coastal marine invertebrates from SE Australia is robust to near-future ocean warming and acidification. Mar. Biol. 157, 2061–2069.
Byrne, M., 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. Oceanogr. Mar. Biol. Annu. Rev. 49, 1–42.
Byrne, M., Przeslawski, R., 2013. Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. Integr. Comp. Biol. 53 (4), 582–596.
Byrne, M., Foo, S., Soars, N.A., Wolfe, K.D.L., Nguyen, H.D., Hardy, N., Dworjanyn, S.A., 2013b. Ocean warming will mitigate the effects of acidification on calcifying sea urchin larvae (Heliocidaris tuberculata) from the Australian global warming hot spot. J. Exp. Mar. Biol. Ecol. 448, 250–257.
Byrne, M., Lamare, M., Winter, D., Dworjanyn, S.A., Uthicke, S., 2013a. The stunting effect of a high CO_2 ocean on calcification and development in sea urchin larvae, a synthesis from the tropics to the poles. Phil. Trans. R Soc. B 368 20120439.
Caldeira, K., Wickett, M., 2005. Ocean models predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. 110 C09S04.
Calosi, P., Rastrick, S.P.S., Graziano, M., Thomas, S.C., Baggini, C., Carter, H.A., Hall-Spencer, J.M., Milazzo, M., Spicer, J.I., 2013. Distribution of sea urchins living near shallow water CO_2 vents is dependent upon species acid-base and ion- regulatory abilities. Mar. Pollut. Bull. 1–15.
Camp, E.F., Schoepf, V., Mumby, P.J., Hardtke, L.A., Rodolfo-Metalpa, R., Smith, D.J., Suggett, D.J., 2018. The future of coral reefs subject to rapid climate change: lessons from natural extreme environments. Front. Mar. Sci. 5, 4. http://dx.doi.org/10.3389/fmars.2018.00004.
Catarino, A.I., Bauwens, M., Dubois, P., 2012. Acid-base balance and metabolic response of the sea urchin *Paracentrotus lividus* to different sea water pH and temperatures. Environ. Sci. Pollut. Res. Int. 19, 2344–2353.

Chan, K.Y.K., Grünbaum, D., O'Donnell, M.J., 2011. Effects of ocean-acidification- induced morphological changes on larval swimming and feeding. J. Exp. Biol. 214, 3857–3867.
Clark, D., Lamare, M., Barker, M., 2009. Response of sea urchin pluteus larvae (Echinodermata: Echinoidea) to reduced seawater pH: a comparison among a tropical, temperate, and a polar species. Mar. Biol. 156, 1125–1137.
Cohen-Rengifo, M., García, E., Hernández, C.A., Hernández, J.C., Clemente, S., 2013. Global warming and ocean acidification affect fertilization and early development of the sea urchin *Paracentrotus lividus*. Cah. Biol. Mar. 54, 667–675.
Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res. 34, 1733–1743.
Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A., 2009. Ocean acidification: the other CO_2 problem. Annu. Rev. Mar. Sci. 1, 169–192.
Dorey, N., Lancon, P., Thorndyke, M.C., Dupont, S., 2013. Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. Global Change Biol. http://dx.doi.org/10.1111/gcb.12276.
Dupont, S., Thorndyke, M., 2013. Direct impacts of near-future ocean acidification on sea urchins. In: Fernández-Palacios, J.M., de Nasciemiento, L., Hernández, J.C., Clemente, S., González, A., Díaz-González, J.P. (Eds.), Climate Change Perspectives from the Atlantic: Past, Present and Future. Servicio de publicaciones de La Universidad de La Laguna, La Laguna, Spain.
Ericson, J.A., Ho, M.A., Miskelly, A., King, C.K., Virtue, P., Tilbrook, B., Byrne, M., 2012. Combined effects of two ocean change stressors, warming and acidification, on fertilization and early development of the Antarctic echinoid Sterechinus neumayeri. Polar Biol. 35, 1027–1034.
Evans, J., Marshall, D., 2005. Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris erythrogramma*. Evolution 59, 106–112.
Fénaux, L., 1968. Maturation des gonades et cycle saisonnier des larves chez *A. lixula, P. lividus* et *P. microtuberculatus* à Villefranche-Sur- Mer. Vie Milieu A Biol. Ma 19, 1–52.
Foo, S.A., Dworjanyn, S.A., Poore, A.G.B., Byrne, M., 2012. Adaptive capacity of the habitat modifying sea urchin Centrostephanus rodgersii to ocean warming and ocean acidification: performance of early embryos. PLoS One 7, e42497.
Fujisawa, H., 1995. Variation in embryonic temperature sensitivity among groups of the sea urchin, Hemicentrotus pulcherrimus, which differ in their habitats. Zool. Sci. 12, 583–589.
García, E., Clemente, S., Hernández, J.C., 2015. Ocean warming ameliorates the negative effects of ocean acidification on *Paracentrotus lividus* larval development and settlement. Mar. Environ. Res. 110, 61–68.
Gattuso, J.P., Hansson, L., 2011. Ocean acidification: background and history. In: Gattuso, J.-P., Hansson, L. (Eds.), Ocean Acidification. Oxford University Press, Oxford, pp. 1–20.
Gianguzza, P., Visconti, G., Gianguzza, F., Sarà, G., Dupont, S., 2014. Temperature modulates the response of the thermophile sea urchin *Arbacia lixula* early life stages to CO_2-driven acidification. Mar. Environ. Res. 93, 70–77.
Girard, D., Herrero, A., Mora, J., Hernández, J., Brito, A., González, N., Catoire, J.L., 2008. Reproductive cycle of the echinoid *Paracentrotus lividus* (Lamarck, 1816) in its southern population limit (Canary Islands eastern Atlantic). Gulf Mex. Sci. 26 (2), 149.
Guidetti, P., Dulcić, J., 2007. Relationships among predatory fish, sea urchins and barrens in Mediterranean rocky reefs across a latitudinal gradient. Mar. Environ. Res. 63, 168–184.
Guillou, M., Michel, C., 1993. Reproduction and growth of *Sphaerechinus granularis* (Echinodermata: Echinoidea) in southern Brittany. J. Mar. Biol. Assoc. U. K. 73, 179–192.
Guillou, M., Lumingas, L.J.L., 1998. Variation in the reproductive strategy of the sea urchin *Sphaerechinus granularis* (Echinodermata: Echinoidea) related to food availability. J. Mar. Biol. Assoc. U. K. 79 (01), 131–136.
Hagström, B., Hagström, B., 1959. The effect of increases and decreased temperatures on fertilization. Exp. Cell Res. 16, 174–183.
Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M., Rowley, S.J., Tedesco, D., Buia, M.-C., 2008. Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature 454, 96–99.
Hall-Spencer, J.M., Allen, R., 2015. The impact of CO_2 emissions on 'nuisance' marine species. Res. Rep. Biodivers. Stud. 4, 33–46.
Hamdoun, A., Epel, D., 2007. Embryo stability and vulnerability in an always changing world. P. Natl. Acad. Sci. USA 104, 1745–1750.
Hardy, N.A., Byrne, M., 2014. Early development of congeneric sea urchins (Heliocidaris) with contrasting life history modes in a warming and high CO_2ocean. Mar. Environ. Res. http://dx.doi.org/10.1016/j.marenvres.2014.07.007.
Harley, C.D.G., Hughes, A.R., Hultgren, K.M., et al., 2006. The impacts of climate change in coastal marine systems. Ecol. Lett. 9, 228–241.
Hernández, J.C., Brito, A., García, N., Gil-Rodríguez, M.C., Herrera, G., Cruz-Reyes, A., Falcón, J.M., 2006. Variación espacial y temporal del índice gonadal de *Diadema antillarum* (Echinodermata: Echinoidea) en las Islas Canarias. Sci. Mar. 70, 689–698.
Hernández, J.C., Clemente, S., Brito, A., 2011. Effects of seasonality on the reproductive cycle of Diadema aff. antillarum in two contrasting habitats: implications for the establishment of a sea urchin fishery. Mar. Biol. http://dx.doi.org/10.1007/s00227-011-1761-0.
Holland, L.Z., Gould-Somero, M., 1982. Fertilization acid of sea urchin eggs: evidence that it is H^+ not CO_2. Dev. Biol. 92, 549–552.
Holland, L.Z., Gould-Somero, M., Paul, M., 1984. Fertilization acid release in Urchins eggs. I. The nature of the acid and the dependence of acid release and egg activation on external pH. Dev. Biol. 103, 337–342.
IPCC, 2014. Climate change 2014: synthesis report. In: Pachauri, R.K., Meyer, L.A. (Eds.), Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland, pp. 151 Core Writing Team.
Johnson, L.G., Babcock, R.C., 1994. Temperature and the larval ecology of the crown-of-thorns starfish, *Acanthaster planci*. Biol. Bull. 168, 419–443.
Kelly, M.W., Padilla, G., Hofmann, G.E., 2013. Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin Strongylocentrotus purpuratus. Global Change Biol. 29, 2536–2546.
Kempf, M., 1962. Recherches *d'écologie* comparée sur *Paracentrotus lividus* (Lmk.) et *Arbacia lixula* (L.). Rec. Trav. Stat. Mar. Endoume. 25, 47–116.
Kurihara, H., Shirayama, Y., 2004. Effects of increased atmospheric CO_2 on sea urchin early development. Mar. Ecol. Prog. Ser. 274, 161–169.
Lewis, E., Wallace, D., 1998. Program Developed for CO_2 System Calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge, Tennessee.
McElroy, D., Nguyen, H.D., Byrne, M., 2012. Respiratory response of the intertidal seastar *Parvulastra exigua* to contemporary and near-future pulses of warming and hypercapnia. J. Exp. Mar. Biol. Ecol. 416–417, 1–7.
Martin, S., Richier, S., Pedrotti, M.Z., Dupont, S., Castejon, C., Gerakis, Y., Kerros, M.E., Oberhänsli, F., et al., 2011. Early development and molecular plasticity in the Mediterranean sea urchin Paracentrotus lividus exposed to CO_2 -driven acidification. J. Exp. Biol. 214, 1357–1368.
Matson, P.G., Yu, P.C., Sewell, M.A., Hofmann, G.E., 2012. Development under elevated CO_2 conditions does not affect lipid utilization and protein content in early life-history stages of the purple sea urchin, *Strongylocentrotus purpuratus*. Biol. Bull. 223, 312–327.
Mehrbach, C., Culberson, C.H., Hawley, J.E., Pytkowicz, R.N., 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907.
Melzner, F., Gutowska, M.A., Langenbuc, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.-O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313–2331.
Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M.A., Bange, H.W., Hanser, H.P., Körtzinger, A., 2013. Future ocean acidification will be amplified 36 by hypoxia in coastal habitats. Mar. Biol. 1875–1888. http://dx.doi.org/10.1007/s00227-012-1954-1.
Mita, M., Hino, A., Yasumasu, I., 1984. Effects of temperature on interactions between eggs and spermatozoa of sea urchin. Biol. Bull. 166, 68–77.
Moulin, L., Catarino, A., Claessens, T., Dubois, P., 2011. Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck, 1816). Mar. Pollut. Bull. 62, 48–54.
Nguyen, H.D., Doo, S., Soars, N.A., Byrne, M., 2012. Noncalcifying larvae in a changing ocean: warming, not acidification/hypercapnia, is the dominant stressor on development of the sea star Meridiastra calcar. Global Change Biol. 18, 2466–2476.
O'Conner, C., Mulley, J.C., 1977. Temperature effects on periodicity and embryology, with observations on the population genetics, of the aquacultural echinoid Heliocidaris tuberculata. Aquaculture 12, 99–114.
O'Connor, M.I., Bruno, J.F., Gaines, S.D., Halpern, B.S., Lester, S.E., Kinlan, B.P., Weiss, J.M., 2007. Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. Proc. Natl. Acad. Sci. U.S.A. 104, 1266–1271.
Padilla-Garmiño, J.L., Kelly, M.W., Evans, T.G., Hofmann, G.E., 2013. Temperature and CO_2 additively regulate physiology, morphology and genomic responses of larval sea urchins, *Strongylocentrotus purpuratus*. Proc. R Soc. Lond B 280 20130155.
Peaucellier, G., Doree, M., 1981. Acid release at activation and fertilization of starfish oocytes. Dev. Growth Differ. 23, 287–296.
Privitera, D., Noli, M., Falugi, C., Chiantore, M., 2011. Benthic assemblages and temperature effects on *Paracentrotus lividus* and *Arbacia lixula* larvae and settlement. J. Exp. Mar. Biol. Ecol. 407, 6–11.
Rasband, W.S., 1997-2012. ImageJ. US National Institutes of Health, Bethesda, Maryland USA Available at: http://imagej.nih.gov/ij/.
Reitzel, A., Miner, B., McEdwar, L., 2004. Relationships between spawning and larval time for benthic marine invertebrates. Mar. Ecol. Prog. Ser. 280, 13–23.
Rodríguez, A, Hernández, J.C., Clemente, S., Coppard, S.E., 2013. A new species of Diadema (Echinodermata: Echinoidea: Diadematidae) from the eastern Atlantic Ocean and a neotype designation of Diadema antillarum (Philippi, 1845). Zootaxa 3636, 144–170.
Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K., Bullister, J.L., Wanninkhof, R., Wong, C.S., Wallace, D.W.R., Tilbrook, B., Millero, F.J., Peng, T.H., Kozyr, A., Ono, T., Rios, A.F., 2004. The oceanic sink for anthropogenic CO_2. Science 305, 367–371.
Sheppard-Brennand, H., Soars, N., Dworjanyn, S., Davis, A., Byrne, M., 2010. Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin *Tripneustes gratilla*. PLoS One 5, e11372.
Small, D.P., Milazzo, M., Bertolini, C., Graham, H., Hauton, C., Hall-Spencer, J.M., Rastrick, S.P.S., 2015. Temporal fluctuations in seawater pCO_2 may be as important as mean differences when determining physiological sensitivity in natural systems. ICES (Int. Counc. Explor. Sea) J. Mar. Sci. http://dx.doi.org/10.1093/icesjms/fsv232.
Stavroff, L.S., 2014. Effects of Ocean Acidification Combined with Multiple Stressors on Early Life Stages of the Pacific Purple Sea Urchin. Master Thesis. Royal Roads University, Canada.
Strathmann, R., 1971. The feeding behavior of planktotrophic echinoderm larvae: mechanisms, regulation, and rates of suspension feeding. J. Exp. Mar. Biol. Ecol. 6, 109–160.
Stumpp, M., Dupont, S., Thorndyke, M.C., Melzner, F., 2011. CO_2 induced seawater acidification impacts sea urchin larval development II: gene expression patterns in pluteus larvae. Comp. Biochem. Phys. A 160 (3), 320–330.
Talmage, S.C., Gobler, C.J., 2009. The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria*

mercenaria), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). Limnol. Oceanogr. 54, 2072–2080.
Talmage, S.C., Gobler, C.J., 2011. Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of northwest Atlantic bivalves. PLoS One 6, e26941.
Turley, C., Keizer, T., Williamson, P., Gattuso, J.-P., Ziveri, P., Monroe, R., Boot, K., Huelsenbeck, M., 2013. Hot, Sour and Breathless – Ocean under stress. Plymouth Marine Laboratory, UK Ocean Acidification Research Programme, European Project on Ocean Acidification, Mediterranean Sea Acidification in a Changing Climate Project. Scripps Institution of Oceanography at UC San Diego OCEANA 6 ISBN: 978-0-9519618-6-5.
Wangensteen, O., 2013. Biology and Phylogeography of the Black Sea Urchin Arbacia Lixula (Echinoidea: Arbacioida). PhD dissertation. Universitat de Barcelona, Spain.
Wangensteen, O., Dupont, S., Casties, I., Turon, X., Palacín, C., 2013. Some like it hot: temperature and acidification modulate larval development and settlement of the sea urchin *Arbacia lixula*. J. Exp. Mar. Biol. Ecol. 449, 304–311.
Wittmann, A., Pörtner, H.O., 2013. Sensitivities of extant animal taxa to ocean acidification. Nat. Clim. Change 3, 995–1001. http://dx.doi.org/10.1038/NCLIMATE1982.
Wolfe, K., Dworjanyn, S., Byrne, M., 2013. Effects of ocean warming and acidification on survival, growth and skeletal development in the early benthic juvenile sea urchin (Heliocidaris erythrogramma). Global Change Biol. 19. http://dx.doi.org/10.1111/gcb.12249.
Yamada, K., Mihashi, K., 1998. Temperature-independent period immediately after fertilization in sea urchin eggs. Biol. Bull. 195, 107–111.